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#13

INVESTIGATIONS IN FISH CONTROL

9. Relation of Chemical Structure to Fish Toxicity in Nitrosalicylanilides and Related Compounds

10. Evaluation of p, p′-DDT as a Reference Toxicant in Bioassays

11. Evaluation of an Electronic Method of Measuring Hematocrits of Fish

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INVESTIGATIONS IN FISH CONTROL

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Fish Control Laboratories
Bureau of Sport Fisheries and Wildlife
U.S. Department of the Interior
P. O. Box 862
La Crosse, Wisconsin 54602

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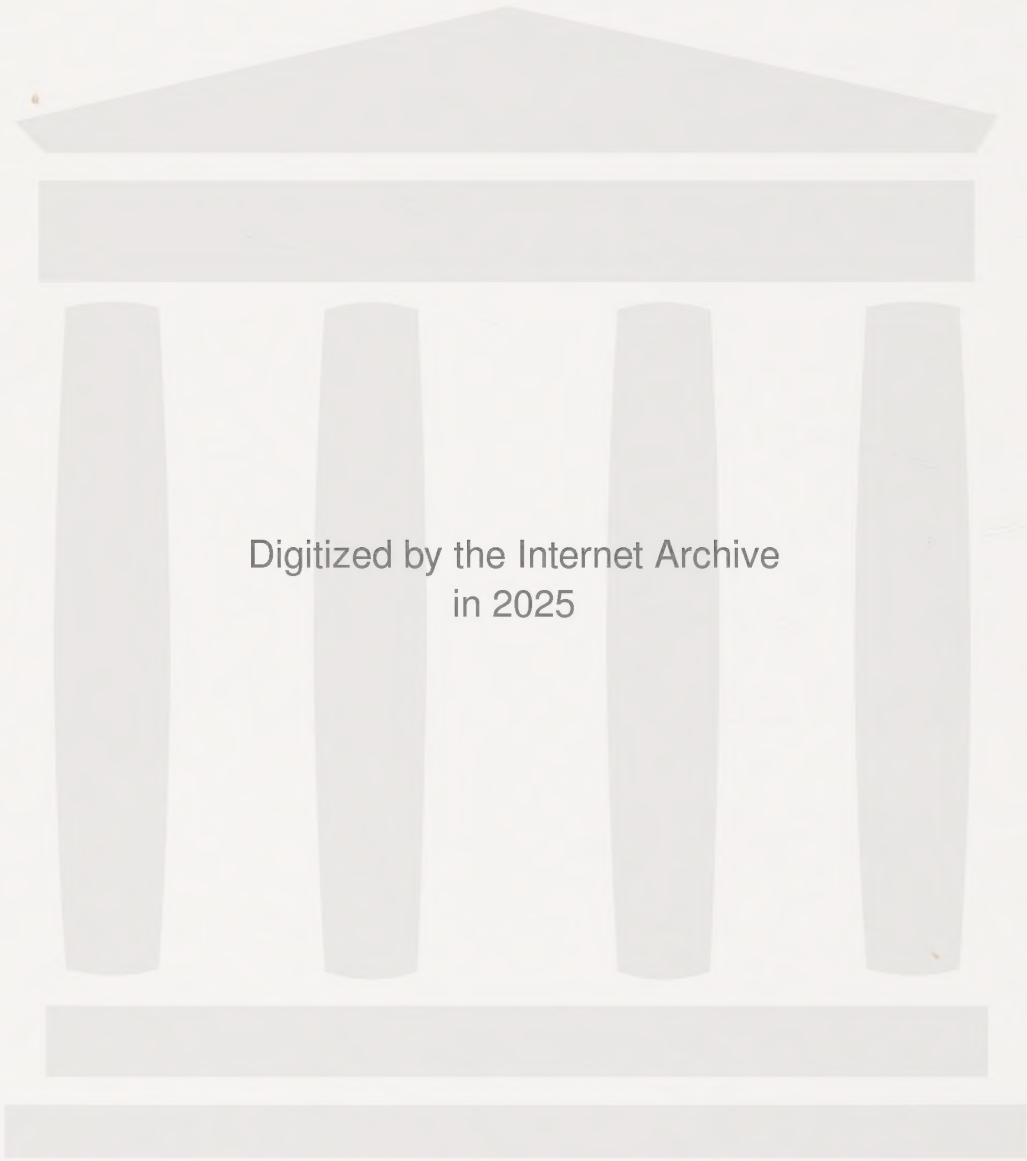
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9. Relation of Chemical Structure to Fish Toxicity in Nitrosalicylanilides and Related Compounds

By Charles R. Walker, Chemist
Fish Control Laboratory
Bureau of Sport Fisheries and Wildlife
La Crosse, Wisconsin

Roland J. Starkey, Microbiologist
Ben Venue Laboratories, Inc.
Bedford, Ohio

Leif L. Marking, Chemist
Fish Control Laboratory
Bureau of Sport Fisheries and Wildlife
La Crosse, Wisconsin

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RELATION OF CHEMICAL STRUCTURE TO FISH TOXICITY IN NITROSALICYLANILIDES AND RELATED COMPOUNDS

By Charles R. Walker, Chemist
Fish Control Laboratory, La Crosse, Wis.

Roland J. Starkey, Microbiologist
Ben Venue Laboratories, Bedford, Ohio

and Leif L. Marking, Chemist
Fish Control Laboratory, La Crosse, Wis.

ABSTRACT.--Relations between chemical structures of salicylanilides and benzanilides and their toxicity to rainbow trout and goldfish were evaluated in standard, static bioassays. Single and multiple substitutions of alkyl-, nitro-, and halo- groups were tested. Substitution of hydroxy- at ortho (2) accompanied by nitro- at meta (3) or sub-meta (5) on the benzoic acid moiety are basic requirements for toxic activity against fish. Halogenation at para (4') markedly affects the selective toxicity of the compounds to either rainbow trout or goldfish.

A search for potent piscicides is a primary function of the Fish Control Laboratory at La Crosse, Wis., and the Southeastern Fish Control Laboratory at Warm Springs, Ga. Candidate compounds are bioassayed to determine their potentials as selective or general fish toxicants. Among the more toxic compounds studied to date is antimycin A (Walker, Lennon, and Berger, 1964). Strong (1958) described the capacity of antimycin A to inhibit the electron-transport system in higher animals, and Derse and Strong (1963) suggested that the antibiotic may be useful as a toxicant highly selective to fish. Since antimycin A is a 3-formamidosalicylic lactone, interest was stimulated in the chemical and biological activities of N-substituted nitrosalicylates and formidosalicylates. Dickie et al. (1963) demonstrated that certain nitro- and formido-substitutions were significantly more active biologically than others. We elected to investigate certain salicylic acid derivatives to detect relations between chemical structure and biological activity against fish.

Investigations with other test organisms have shown some relation between chemical structure of salicylanilides and toxicity. Taborsky et al. (1959, 1962, 1963) described the importance of specific substitutions to antimicrobial activity. Baichwal et al. (1960a) described fungicidal activities, and Schraufstätter (1962) demonstrated relations between structure and molluscicidal properties. Recently, Starkey and Howell (1965) pointed out the importance of molecular structure of salicylanilides to selective toxicity to the sea lamprey (Petromyzon marinus).

The object of this study was to ascertain the structural requirements of compounds to produce toxicity in two fishes. The compounds included certain substituted nitrosalicylanilides and benzanilides with consideration given to single and multiple substituents, and in particular to the locus of the nitro and halogen groups. The fish were rainbow trout (Salmo gairdneri) and goldfish (Carassius auratus).

METHODS AND MATERIALS

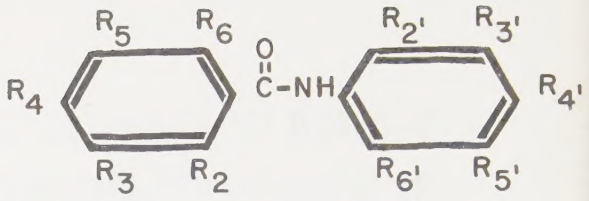
The bioassay methods used were described by Lennon and Walker (1964) for the evaluation of fish control agents. All bioassays were conducted in reconstituted water at 12°C. The rainbow trout were acquired from the Manchester, Iowa, National Fish Hatchery and acclimated to the laboratory water supply. The mean LC_{50} of p,p'-DDT for them was 6.8 parts per billion, with a range from 1.9 to 14.0 p.p.b. (table 1). The goldfish were obtained from the Lake Mills, Wis., National Fish Hatchery and weighed from 0.7 to 2.5 grams. The mean LC_{50} of p,p'-DDT for them was 41.2 p.p.b., with a range from 27.0 to 76.0 p.p.b. (table 1).

TABLE 1.--Comparative sensitivities of different lots of fish to the reference standard p,p'-DDT expressed as the LC_{50} value in p.p.b.

Species and lot	Average weight (grams)	Date bioassayed	LC_{50} (p.p.b.)
Rainbow trout:			
Lot 16a	0.92	Mar. 24, 1964	14.0
Lot 17	0.92	Apr. 10, 1964	4.6
Lot 18C	0.20	May 21, 1965	1.9
Mean LC_{50}			6.8
Goldfish:			
Lot 1	2.50	Mar. 4, 1964	76.0
Lot 19	2.38	Mar. 31, 1964	27.0
Lot 185	0.70	Apr. 3, 1964	32.0
		June 27, 1965	29.7
Mean LC_{50}			41.2

Toxicity of the compounds was determined at three concentrations (0.1, 1.0, and 10 parts per million) on rainbow trout and goldfish in 2.5- or 15-liter glass containers. Ten fish were bioassayed at each concentration, and the effects of the chemicals upon the fish were recorded at 0.25, 0.5, 1, 3, 24, 48, 72, and 96 hours. The data presented in this paper are confined to observations made at the end of 3 and 48 hours, which appeared to be the critical times for differentiating the gross acute toxic effects.

Ben Venue Laboratories, Inc., Bedford, Ohio, furnished substituted salicylanilides and benzanilides for evaluation of relations between structure and piscicidal activity (fig. 1 and table 2). Substituted nitrosalicylanilides of the general formula depicted in figure 1 have been documented (Taborsky et al., 1959; Taborsky and Starkey, 1963; and an unpublished manuscript "Some substituted salicylanilides"



SALICYLANILIDES

R_2 = OH accompanied by one or more of the following:

R_3 = NO_2, and/or R_5 = NO_2, Cl, Br, or acetylamine

$R_{2'}-R_{6'}$ = NO_2, F, Cl, Br, I, CH_3, OCH_3, C_2H_5, and/or phenylazo in one or more positions.

BENZANILIDES

R_2-R_5 = NO_2, Cl, and/or OH with the following:

$R_{2'}-R_{6'}$ = NO_2, Cl, Br, I, CH_3, OCH_3, and C_2H_5 in one or more positions.

Figure 1.--General structure of substituted salicylanilides and benzanilides.

by Roland J. Starkey). They can be arbitrarily divided into five groups based upon substituents in the aniline moiety: (1) halonitrosalicylanilides; (2) halonitrosalicylotoluidides; (3) nitrosalicyloxylidides; (4) nitrosalicylanisidides; and (5) a miscellaneous group including 4'-phenylazo-3-nitrosalicylanilide and 2'-ethyl-3-nitrosalicylanilide.

Salicylanilides are synthesized by the condensation of salicylic acid or a derivative with aniline or a derivative thereof. This produces a biphenyl molecule with a CONH bridge and is characterized by the presence of an ortho phenolic hydroxyl group (OH) in the acid moiety. Benzanilides, also biphenyl in structure with the CONH bridge but devoid of the hydroxyl group, are prepared in the same manner with a derivative of benzoic acid.

3-nitrosalicylanilides, in addition to a hydroxyl group, possess a nitro (NO_2) group at the 3 (meta) position of the acid moiety

TABLE 2.--Compounds used in evaluating the relation of chemical structure to fish toxicity

Compound	Chemical name[1]	Compound	Chemical name[1]
1.	3-nitrosalicylanilide	36.	2'-nitro-p-salicylanisidide
2.	2'-iodo-3-nitrosalicylanilide	37.	2',3-dinitro-m-salicylanisidide
3.	2'-ethyl-3-nitrosalicylanilide	38.	2'-chloro-3,4'-dinitrosalicylanilide
4.	3'-chloro-3-nitrosalicylanilide	39.	3-nitrosalicylanilide
5.	3'-bromo-3-nitrosalicylanilide	40.	benzanilide
6.	3'-iodo-3-nitrosalicylanilide	41.	3'-chloro-3-hydroxybenzanilide
7.	4'-chloro-3-nitrosalicylanilide	42.	4'-chloro-3-hydroxybenzanilide
8.	4'-bromo-3-nitrosalicylanilide	43.	2'-chloro-2-nitrobenzanilide
9.	4'-iodo-3-nitrosalicylanilide	44.	3'-chloro-2-nitrobenzanilide
10.	4'-phenylazo-3-nitrosalicylanilide	45.	4'-chloro-2-nitrobenzanilide
11.	2'-chloro-5-nitrosalicylanilide	46.	4'-bromo-2-nitrobenzanilide
12.	3'-fluoro-5-nitrosalicylanilide	47.	3'-chloro-2-nitro-o-benzotoluidide
13.	4'-chloro-5-nitrosalicylanilide	48.	3-nitrobenzanilide
14.	4'-iodo-5-nitrosalicylanilide	49.	2'-chloro-3-nitrobenzanilide
15.	3'-chloro-5-acetamidosalicylanilide	50.	3'-chloro-3-nitrobenzanilide
16.	2',4',6'-trichloro-3-nitrosalicylanilide	51.	3'-chloro-3-nitro-p-benzotoluidide
17.	3',4'-dichloro-3-nitrosalicylanilide	52.	2'-chloro-4-nitrobenzanilide
18.	2',5'-dibromo-3-nitrosalicylanilide	53.	3'-chloro-4-nitrobenzanilide
19.	4'-chloro-5-bromo-3-nitrosalicylanilide	54.	5'-chloro-4-nitrobenzanilide
20.	4',5-dibromo-3-nitrosalicylanilide	55.	p-chlorobenzanilide
21.	3-nitro-2',3'-salicyloxylidide	56.	3,5-dinitrobenzanilide
22.	3-nitro-2',4'-salicyloxylidide	57.	2'-chloro-3,5-dinitrobenzanilide
23.	3-nitro-2',5'-salicyloxylidide	58.	3'-chloro-3,5-dinitrobenzanilide
24.	3-nitro-2',6'-salicyloxylidide	59.	3'-bromo-3,5-dinitrobenzanilide
25.	3'-chloro-3-nitro-o-salicylotoluidide	60.	4'-bromo-3,5-dinitrobenzanilide
26.	4'-chloro-3-nitro-o-salicylotoluidide	61.	4'-iodo-3,5-dinitrobenzanilide
27.	2'-chloro-3-nitro-p-salicylotoluidide	62.	2'-fluoro-3,5-dinitrobenzanilide
28.	6'-chloro-3-nitro-o-salicylotoluidide	63.	3,5-dinitro-o-benzotoluidide
29.	3,5'-dinitro-o-salicylotoluidide	64.	3,5-dinitro-2',3'-benzoxylidide
30.	2',3-dinitro-p-salicylotoluidide	65.	3'-chloro-3,5-dinitro-p-benzotoluidide
31.	4'-bromo-3-nitro-o-salicylotoluidide	66.	5'-chloro-3,5-dinitro-o-benzotoluidide
32.	5'-chloro-3-nitro-o-salicylanisidide	67.	3'-chloro-3,5-dinitro-o-benzotoluidide
33.	4'-chloro-2',5'-dimethoxy-3-nitrosalicylanilide	68.	3,3',5-trinitrobenzanilide
34.	5'-methyl-o-salicylanisidide	69.	2',3,4',5-tetranitrobenzanilide
35.	4'-nitro-o-salicylanisidide	70.	5'-chloro-3,5-dinitro-o-benzanisidide
		71.	2',5'-diethyl-3,5-dinitrobenzanilide

[1] A.C.S. or I.U.C. nomenclature

(fig. 1 and table 2). 5-nitrosalicylanilides differ with the locus of the nitro group being oriented to the five position. In several instances halogens have also been substituted at the 5 position to produce 5-chloro-3-nitrosalicylanilides or 5-bromo-3-nitrosalicylanilides. Variations in these compounds have been achieved by introducing single (mono) or multiple (poly) substituents in the anilide portion of the molecule. Diversity was attained with isomers by changing the locus of one or all of the substituents. In one case (compound 15, table 2) the 5-substituted nitro group has been reduced to an acetamido group.

RESULTS

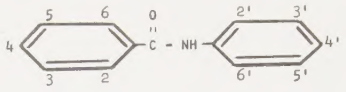
NITROSALICYLANILIDES

Mono-substituted nitrosalicylanilides.--This series, with the exception of 2'-ethyl-3-nitrosalicylanilides, 2'-chloro-5-nitrosalicylanilide, and the 5-acetamido- derivative of 3'-chloro-5-nitrosalicylanilide (compounds 3, 11, 15, table 3), effected a complete kill of rainbow trout during a 48-hour test period at 1 p.p.m. Most of the substituted 3-nitrosalicylanilides at this concentration were lethal within 3 hours. 3-nitrosalicylanilide per se (compound 1, table 3) did not share this property of producing death quickly. Substituted 5-nitrosalicylanilides did not kill fish quickly at less than 10 p.p.m. 4'-phenylazo-3-nitrosalicylanilide and 4'-iodo-3-nitrosalicylanilide (compounds 9 and 10, table 3) were the only mono-halonitrosalicylanilides lethal to trout at 0.1 p.p.m.

Seven of the mono-substituted nitrosalicylanilides (compounds 6-10 and 13-14, table 3) killed all rainbow trout and goldfish at 10 p.p.m. Two of them, 4'-chloro-3-nitrosalicylanilide and 4'-iodo-3-nitrosalicylanilide, were effective within 3 hours. 4'-chloro-3-nitrosalicylanilide was lethal to goldfish at 0.1 p.p.m. but not to rainbow trout during the 48-hour test. Seven others (Nos. 1, 2, 4, 5, 11, 12, and 15) were lethal to trout at 1 p.p.m. but varied in toxicity to goldfish.

The toxicity of mono-substituted nitrosalicylanilides to goldfish increased as the locus of the substituent in the aniline moiety was

TABLE 3.--Relation of chemical structure to piscicidal activity of mono-substituted nitrosalicylanilides to two species of fish at 12°C.

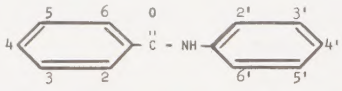


Compound	Locus of substituent on the structure										Toxicity of the compound to--					
											Rainbow trout at--			Goldfish at--		
	2	3	4	5	6	2'	3'	4'	5'	6'	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.
1.	OH	NO_2	--	--	--	--	--	--	--	--	0	10	[1] 10	0	0	10
2.	OH	NO_2	--	--	--	I	--	--	--	--	0	10	[1] 10	0	0	[1] 10
3.	OH	NO_2	--	--	--	C_2H_5	--	--	--	--	0	0	--	0	0	--
4.	OH	NO_2	--	--	--	--	Cl	--	--	--	0	10	[1] 10	0	6	[1] 10
5.	OH	NO_2	--	--	--	--	Br	--	--	--	0	[1] 10	[1] 10	0	9	[1] 10
6.	OH	NO_2	--	--	--	--	I	--	--	--	0	[1] 10	[1] 10	0	10	[1] 10
7.	OH	NO_2	--	--	--	--	--	Cl	--	--	0	[1] 10	[1] 10	10	[1] 10	[1] 10
8.	OH	NO_2	--	--	--	--	--	Br	--	--	0	[1] 10	[1] 10	0	10	[1] 10
9.	OH	NO_2	--	--	--	--	--	I	--	--	9	[1] 10	[1] 10	0	[1] 10	[1] 10
10.	OH	NO_2	--	--	--	--	--	PA[2]	--	--	10	[1] 10	[1] 10	0	10	10
11.	OH	--	--	NO_2	--	Cl	--	--	--	--	0	8	[1] 10	1	1	[1] 10
12.	OH	--	--	NO_2	--	--	F	--	--	--	0	10	[1] 10	0	1	10
13.	OH	--	--	NO_2	--	--	--	Cl	--	--	0	10	[1] 10	1	10	[1] 10
14.	OH	--	--	NO_2	--	--	--	I	--	--	0	10	[1] 10	1	10	[1] 10
15.	OH	--	--	AcAm[3]	--	--	Cl	--	--	--	1	9	[1] 10	0	1	10

[1] All dead at 3 hours.
[2] Phenyl-azo.
[3] Acetamido.

oriented from ortho (2') to para (4'). For example, although iodo-3-nitrosalicylanilides were evaluated at all three positions, only 2'-iodo-3-nitrosalicylanilide was nontoxic to goldfish at 1 p.p.m. 3'-iodo- and 4'-iodo-3-nitrosalicylanilide are lethal to both species at this concentration, but 4'-iodo-3-nitrosalicylanilide was lethal to both species within 3 hours. In addition the latter compound killed 9 out of 10 rainbow trout at 0.1 p.p.m., but the ortho (2') and meta (3') isomers were ineffective. Likewise, 3'-chloro-3-nitrosalicylanilide required 48 hours to kill 6 out of 10 goldfish and all of the rainbow trout at 1 p.p.m. 4'-chloro-3-nitrosalicylanilide produced total kills of both species within 3 hours. Further trials revealed that 4'-chloro-3-nitrosalicylanilide is far more toxic to goldfish than to rainbow trout at 0.1 p.p.m. (tables 4 and 5).

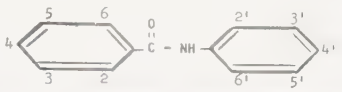
TABLE 4.--Relation of chemical structure of nitrosalicylanilides to selective piscicidal activity for rainbow trout and goldfish at 12°C.



Compound	Locus of substituent on the structure										Toxicity of the compound to--					
											Rainbow trout at--			Goldfish at--		
	2	3	4	5	6	2'	3'	4'	5'	6'	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.
10.	OH	NO_2	--	--	--	--	--	PA[2]	--	--	10	[1] 10	[1] 10	0	10	10
19.	OH	NO_2	--	Br	--	--	--	Cl	--	--	10	[1] 10	[1] 10	3	10	[1] 10
20.	OH	NO_2	--	Br	--	--	--	Br	--	--	10	[1] 10	[1] 10	2	10	[1] 10
9.	OH	NO_2	--	--	--	--	--	I	--	--	9	10	[1] 10	0	10	[1] 10
31.	OH	NO_2	--	--	--	CH_3	--	Br	--	--	2	[1] 10	[1] 10	0	10	[1] 10
17.	OH	NO_2	--	--	--	--	Cl	Cl	--	--	1	[1] 10	[1] 10	1	10	[1] 10
8.	OH	NO_2	--	--	--	--	--	Br	--	--	0	10	[1] 10	0	[1] 10	[1] 10
6.	OH	NO_2	--	--	--	--	I	--	--	--	0	[1] 10	[1] 10	0	10	[1] 10
13.	OH	--	--	NO_2	--	--	--	Cl	--	--	0	10	[1] 10	1	10	[1] 10
14.	OH	--	--	NO_2	--	--	--	I	--	--	0	10	[1] 10	1	10	[1] 10
33.	OH	NO_2	--	--	--	CH_3O	--	Cl	CH_3O	--	0	[1] 10	[1] 10	1	10	[1] 10
7.	OH	NO_2	--	--	--	--	--	Cl	--	--	0	[1] 10	[1] 10	10	[1] 10	[1] 10

[1] All dead at 3 hours.
[2] C_6H_5-N=N-(phenyl-azo).

TABLE 5.--Obligatory structural requirements for 4'-chloro-3-nitrosalicylanilide as a selective piscicidal agent for goldfish and rainbow trout



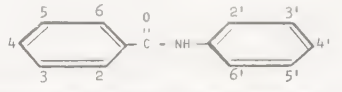
Compound	Locus of substituent on the structure										Toxicity of the compound to--					
											Rainbow trout at--			Goldfish at--		
	2	3	4	5	6	2'	3'	4'	5'	6'	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.
7.	OH	NO_2	--	--	--	--	--	Cl	--	--	0	[1] 10	[1] 10	10	[1] 10	[1] 10
17.	OH	NO_2	--	--	--	--	Cl	Cl	--	--	1	[1] 10	[1] 10	1	10	[1] 10
39.	OH	NO_2	--	--	--	--	--	--	--	--	0	0	10	0	0	10
4.	OH	NO_2	--	--	--	--	Cl	--	--	--	0	10	[1] 10	0	6	10
41.	--	OH	--	--	--	--	Cl	--	--	--	0	0	10	0	0	7
44.	NO_2	--	--	--	--	--	Cl	--	--	--	0	0	10	0	0	6
50.	--	NO_2	--	--	--	--	Cl	--	--	--	0	2	[1] 10	0	0	10
53.	--	--	NO_2	--	--	--	Cl	--	--	--	0	0	0	0	0	0
58.	--	NO_2	--	NO_2	--	--	Cl	--	--	--	0	0	7	0	0	0
42.	--	OH	--	--	--	--	--	Cl	--	--	0	0	10	0	0	2
45.	NO_2	--	--	--	--	--	--	Cl	--	--	0	0	10	0	0	5
54.	--	--	NO_2	--	--	--	--	Cl	--	--	0	0	10	0	0	6

[1] All dead at 3 hours.

Poly-substituted nitrosalicylanilides.--Tests were made to determine whether toxicity and selectivity could be enhanced with poly-substituted derivatives of 3-nitrosalicylanilide. This series includes compounds with multiple substituents, some containing a halogen and a methyl group (CH_3) in the aniline moiety (halo-3-nitrosalicylotoluidides), some with two methyl groups (3'-nitrosalicyloxylidides), and others with halogenated-methoxy (CH_3O) derivatives (halo-3-nitrosalicylanisidides) (table 6).

3',4'-dichloro-3-nitrosalicylanilide (compound 17, tables 2, 4, and 6) killed all goldfish and rainbow trout at 1 p.p.m., but 9 out

TABLE 6.--Relation of chemical structure to piscicidal activity of poly-substituted nitrosalicylanilides to two species of fish at 12°C.



Compound	Locus of substituent on the structure										Toxicity of the compound to--					
											Rainbow trout at--			Goldfish at--		
	2	3	4	5	6	2'	3'	4'	5'	6'	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.
16.	OH	NO_2	--	--	--	Cl	--	Cl	--	Cl	0	0	[1] 10	0	0	10
17.	OH	NO_2	--	--	--	--	Cl	Cl	--	--	1	[1] 10	[1] 10	1	10	[1] 10
18.	OH	NO_2	--	--	--	Br	--	--	Br	--	0	10	[1] 10	0	3	[1] 10
19.	OH	NO_2	--	Br	--	--	--	Cl	--	--	10	[1] 10	[1] 10	3	10	[1] 10
20.	OH	NO_2	--	Br	--	--	--	Br	--	--	10	[1] 10	[1] 10	2	10	[1] 10
21.	OH	NO_2	--	--	--	CH_3	CH_3	--	--	--	0	10	[1] 10	2	0	10
22.	OH	NO_2	--	--	--	CH_3	--	CH_3	--	--	0	10	[1] 10	0	1	[1] 10
23.	OH	NO_2	--	--	--	CH_3	--	--	CH_3	--	0	9	[1] 10	0	0	10
24.	OH	NO_2	--	--	--	CH_3	--	--	--	CH_3	0	1	10	0	0	10
25.	OH	NO_2	--	--	--	CH_3	Cl	--	--	--	1	10	[1] 10	0	2	10
26.	OH	NO_2	--	--	--	CH_3	--	Cl	--	--	0	[1] 10	[1] 10	0	10	[1] 10
27.	OH	NO_2	--	--	--	Cl	--	CH_3	--	--	1	[1] 10	[1] 10	1	[1] 10	[1] 10
28.	OH	NO_2	--	--	--	CH_3	--	--	--	Cl	0	0	10	0	0	0
29.	OH	NO_2	--	--	--	CH_3	--	--	NO_2	--	0	0	[1] 10	0	0	9
30.	OH	NO_2	--	--	--	NO_2	--	CH_3	--	--	0	10	[1] 10	0	0	10
31.	OH	NO_2	--	--	--	CH_3	--	Br	--	--	2	[1] 10	[1] 10	0	10	[1] 10
32.	OH	NO_2	--	--	--	CH_3O	--	--	Cl	--	0	[1] 10	[1] 10	0	1	[1] 10
33.	OH	NO_2	--	--	--	CH_3O	--	Cl	CH_3O	--	0	[1] 10	[1] 10	1	10	[1] 10
34.	OH	NO_2	--	--	--	CH_3O	--	--	CH_3	--	0	9	10	1	0	10
35.	OH	NO_2	--	--	--	CH_3O	--	NO_2	--	--	0	2	[1] 10	1	0	10
36.	OH	NO_2	--	--	--	NO_2	--	CH_3O	--	--	0	8	[1] 10	0	4	[1] 10
37.	OH	NO_2	--	--	--	NO_2	--	--	CH_3O	--	0	10	[1] 10	0	0	0
38.	OH	NO_2	--	--	--	Cl	--	NO_2	--	--	0	[1] 10	[1] 10	2	10	[1] 10
39.	OH	NO_2	--	--	--	--	--	--	--	--	0	0	10	0	0	10

[1] All dead at 3 hours.

of 10 of each species survived at 0.1 p.p.m. Compared with 4'-chloro-3-nitrosalicylanilide, this compound was less toxic to goldfish but more toxic to rainbow trout. Also, 2',4',6'-trichloro-3-nitrosalicylanilide (compound 16, table 6) was toxic to goldfish and rainbow trout at 10 p.p.m. but not at 1 p.p.m. It thus appears that the receptors of goldfish, sensitive to 4'-chloro-3-nitrosalicylanilide, cannot easily accommodate bulkier molecules irrespective of a common para (4') substituent. A similar observation on the sea lamprey was made by Starkey and Howell (1965).

A change in locus of the nitro- group from meta (3) to sub-meta (5) on the salicylic acid moiety of para (4') halogenated anilides resulted in slightly more toxicity to goldfish than rainbow trout at 0.1 p.p.m. (compounds 13 and 14, table 6). The toxicity of 5-nitrosalicylanilides to rainbow trout and goldfish was reduced when the halogen was moved to the meta (3') position. The ortho (2') substitution was even less toxic.

Among the poly-substituted 3-nitrosalicylanilides, 4',5-dibromo-3-nitrosalicylanilide and 4'-bromo-5-chloro-3-nitrosalicylanilide (compounds 19-20, tables 4 and 6) were lethal to rainbow trout at 0.1 p.p.m. but only slightly toxic to goldfish. At 1 p.p.m. both compounds were lethal to trout within 3 hours whereas goldfish succumbed later during the 48-hour test.

The toxicity of 3-nitrosalicyloxylidides (compounds 21-24, table 6) to rainbow trout varies with the loci of the methyl (CH_3) substituents in the aniline moiety. Each compound in this series has an ortho (2') methyl group in common but differs with the ring position of the second substituent either at the meta (3'), para (4'), sub-meta (5'), or sub-ortho (6') positions. The 2',3'- and 2',4'-isomers are completely lethal to rainbow trout at 1 p.p.m. In comparison, the 2',5'- and 2',6'- isomers produce 90- and 10-percent mortality, respectively. None of the isomers was toxic to goldfish except at 10 p.p.m. A similar variation in the toxicity of 3-nitrosalicyloxylidides of the sea lamprey was reported by Starkey and Howell (1965).

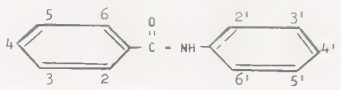
Halo-3-nitrosalicylotoludides were tested to ascertain if the activity of 3-nitrosalicyloxylidides is significantly altered by substituting a halogen, nitro, or methoxy group for a methyl group (table 7). 3-nitro-2,3-salicyloxylidide and 3-nitro-2,4-salicyloxylidide (compounds 21 and 22) were not toxic to goldfish at 1 p.p.m. Substitution of the meta (3') and para (4') with chlorine atoms, however, produced compounds (3'-chloro-3-nitro-o-salicylotoluidide and 4'-chloro-3-nitro-o-salicylotoluidide, compounds 25 and 26) which were lethal at 1 p.p.m. but not at 0. 1 p.p.m. Substitution of the ortho (2') methyl group with a nitro group, as in the case of 2',3-dinitro-p-salicylotoluidide (compound 30, table 7), resulted in a compound which was nontoxic to goldfish at 1 p.p.m.

Other compounds in which one of the methyl groups was replaced by a methoxy group (3-nitrosalicylanisidides) were relatively nontoxic to goldfish at 1 p.p.m. The only exception was 2',3-dinitro-p-salicylanisidide (compound 36) which killed 4 of 10 goldfish. Its isomer, 2',3-dinitro-5-salicylanisidide (compound 37), was nontoxic to goldfish at 10 p.p.m. but killed all rainbow trout at 1 p.p.m. Altering the loci of chloro and methyl groups, as in the case of 2'-chloro-3-nitro-6-salicylanisidide (compound 28) eliminated toxicity to goldfish at 10 p.p.m. In no instance did the halo-3-nitrosalicylotoludides, 3-nitrosalicyloxylidides, and 3-nitrosalicylanisidides exhibit selective toxicity to goldfish over rainbow trout. Only one compound, a 2',5'-dimethoxy-3-nitrosalicylanilide, demonstrated a slight toxicity to goldfish at 0. 1 p.p.m. and did not affect rainbow trout.

BENZANILIDES

Benzanilides in this series (table 8), differing from salicylanilides by the absence of an ortho hydroxyl substituent, include benzanilide (compound 40), meta (3) hydroxybenzanilides (compounds 41-42), ortho (2) nitrobenzanilides (compounds 43-47), meta (3) nitrobenzanilides (compounds 48-51), para (4) nitrobenzanilides (compounds 52-54), para

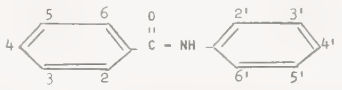
TABLE 7.--Relation of chemical structure to piscicidal activity of 3-nitrosalicyloxylidides, 3-nitrosalicylanisidides, and 3-nitrosalicylotoluidides to two species of fish at 12°C.



	Locus of substituent on the structure										Toxicity of the compound to--					
											Rainbow trout at--			Goldfish at--		
	2	3	4	5	6	2'	3'	4'	5'	6'	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.
21.	OH	NO_2	--	--	--	CH_3	CH_3	--	--	--	0	10	10	2	0	10
25.	OH	NO_2	--	--	--	CH_3	Cl	--	--	--	1	10	[1] 10	0	10	[1] 10
22.	OH	NO_2	--	--	--	CH_3	--	CH_3	--	--	0	10	10	0	1	10
26.	OH	NO_2	--	--	--	CH_3	--	Cl	--	--	0	[1] 10	[1] 10	1	[1] 10	[1] 10
27.	OH	NO_2	--	--	--	Cl	--	CH_3	--	--	1	[1] 10	[1] 10	1	[1] 10	[1] 10
38.	OH	NO_2	--	--	--	Cl	--	NO_2	--	--	0	[1] 10	[1] 10	2	10	[1] 10
30.	OH	NO_2	--	--	--	NO_2	--	CH_3	--	--	0	10	[1] 10	0	0	10
36.	OH	NO_2	--	--	--	NO_2	--	CH_3O	--	--	0	8	[1] 10	0	4	[1] 10
35.	OH	NO_2	--	--	--	CH_3O	--	NO_2	--	--	0	2	[1] 10	1	0	10
29.	OH	NO_2	--	--	--	CH_3	--	--	NO_2	--	0	0	[1] 10	0	0	9
32.	OH	NO_2	--	--	--	CH_3O	--	--	Cl	--	0	[1] 10	[1] 10	1	10	[1] 10
34.	OH	NO_2	--	--	--	CH_3O	--	--	CH_3	--	0	9	10	0	0	0
37.	OH	NO_2	--	--	--	NO_2	--	--	CH_3O	--	0	10	[1] 10	0	0	0
28.	OH	NO_2	--	--	--	Cl	--	--	--	CH_3	0	0	10	0	0	0

[1] All dead at 3 hours.

TABLE 8.--Relation of chemical structure to piscicidal activity of mono- and poly-substituted benzanilides to two species of fish at 12°C.



Compound	Locus of substituent on the structure										Toxicity of the compound to--					
											Rainbow trout at--			Goldfish at--		
	2	3	4	5	6	2'	3'	4'	5'	6'	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.	0.1 p.p.m.	1.0 p.p.m.	10.0 p.p.m.
40.	--	--	--	--	--	--	--	--	--	--	0	0	0	0	0	0
41.	--	OH	--	--	--	--	Cl	--	--	--	0	0	10	0	0	7
42.	--	OH	--	--	--	--	--	Cl	--	--	0	0	10	0	0	2
43.	NO_2	--	--	--	--	Cl	--	--	--	--	0	0	0	0	0	0
44.	NO_2	--	--	--	--	--	Cl	--	--	--	0	0	10	0	0	6
45.	NO_2	--	--	--	--	--	--	Cl	--	--	0	0	10	0	0	5
46.	NO_2	--	--	--	--	--	--	Br	--	--	0	0	10	0	1	2
[2] 47.	NO_2	--	--	--	--	CH_3	Cl	--	--	--	0	0	0	0	0	0
[2] 48.	--	NO_2	--	--	--	--	--	--	--	--	0	0	10	0	0	10
49.	--	NO_2	--	--	--	Cl	--	--	--	--	0	0	10	0	0	1
50.	--	NO_2	--	--	--	--	Cl	--	--	--	0	2	[1] 10	0	0	[1] 10
[2] 51.	--	NO_2	--	--	--	--	Cl	CH_3	--	--	0	0	0	1	0	0
52.	--	--	NO_2	--	--	Cl	--	--	--	--	0	0	0	0	0	0
53.	--	--	NO_2	--	--	--	Cl	--	--	--	0	0	0	0	0	0
54.	--	--	NO_2	--	--	--	--	Cl	--	--	0	0	10	0	0	6
55.	--	--	Cl	--	--	--	--	--	--	--	0	0	0	0	0	0
56.	--	NO_2	--	NO_2	--	--	--	--	--	--	0	0	0	0	0	0
57.	--	NO_2	--	NO_2	--	Cl	--	--	--	--	0	0	0	0	0	0
58.	--	NO_2	--	NO_2	--	--	Cl	--	--	--	0	0	7	0	0	0
59.	--	NO_2	--	NO_2	--	--	Br	--	--	--	0	4	0	0	0	0
60.	--	NO_2	--	NO_2	--	--	--	Br	--	--	[3] 0	0	0	0	0	0
[2] 61.	--	NO_2	--	NO_2	--	--	--	I	--	--	0	0	0	0	0	0
62.	--	NO_2	--	NO_2	--	F	--	--	--	--	0	0	10	0	0	10
[2] 63.	--	NO_2	--	NO_2	--	CH_3	--	--	--	--	0	0	10	0	1	8
64.	--	NO_2	--	NO_2	--	CH_3	--	CH_3	--	--	0	0	1	0	0	0
65.	--	NO_2	--	NO_2	--	--	Cl	CH_3	--	--	0	0	2	0	0	0
66.	--	NO_2	--	NO_2	--	CH_3	--	--	Cl	--	0	0	[1] 10	0	0	0
[2] 67.	--	NO_2	--	NO_2	--	CH_3	Cl	--	--	--	0	0	[1] 10	0	0	0
68.	--	NO_2	--	NO_2	--	--	NO_2	--	--	--	0	0	[1] 10	0	0	0
69.	--	NO_2	--	NO_2	--	NO_2	--	NO_2	--	--	0	0	10	0	0	0
70.	--	NO_2	--	NO_2	--	CH_3O	--	--	Cl	--	0	0	0	0	1	0
71.	--	NO_2	--	NO_2	--	C_2H_5	--	--	--	C_2H_5	[3] 0	[3] 0	0	0	0	1

[1] All dead at 3 hours.
[2] Precipitation at 10 p.p.m.
[3] Sounding.

(4) chlorobenzanilides (compound 55), and 3,5-dinitrobenzanilides (compounds 57-71). As in the substituted salicylanilides, the substituents (mono or poly) are either halogen, methyl, or nitro groups on the anilide portion of the molecule (fig. 1, table 2).

In general, the benzanilides are relatively nontoxic to rainbow trout and goldfish (tables 5 and 8). Only three (compounds 48, 50, and 62) were lethal to goldfish at 10 p.p.m. It appears that substituted benzanilides have minimal toxicity in comparison with salicylanilides. This is attributed to the lack of an ortho hydroxyl substituent. These findings agree with those of Baichwal et al. (1960a) and Starkey and Howell (1965). For example, 3'-chloro-2-nitrobenzanilide and 4'-chloro-2-nitrobenzanilide (compounds 44 and 45, tables 5 and 8) were the most toxic to both species. The speed of activity was increased markedly by moving the nitro group to the meta (3) position in the acid moiety. Substitution of the nitro group at the para (4) position produced compounds intermediate in toxicity.

The 3,5-dinitrobenzanilides were relatively insoluble at 10 p.p.m. and were therefore difficult to bioassay. They were, with several exceptions, selective to rainbow trout at 10 p.p.m. (table 5). Only two (compounds 62 and 63) were significantly toxic to goldfish at this concentration. 3,3',5-trinitrobenzanilide was more toxic than 2',3,4',5-tetranitrobenzanilide (compounds 68 and 69) and may reflect the effects of saturation on both toxicity and selectivity.

Another group of benzanilides (compounds 41, 44, 50, 53, and 58) possess a meta (3') chloro-substituent and vary only in the locus of a substituent in the acid moiety (table 8). Of them, compounds 44, 50, 53, and 58 are various substituted nitrobenzanilides and compound 41 is a substituted 3-hydroxybenzanilide. None has the ortho hydroxyl substituent characteristic of salicylanilides. Compound 53 which has the nitro substituted in the para (4) position was nontoxic to both fishes. The other compounds (41, 44, 50, and 58) were lethal to rainbow trout at 10 p.p.m., but only compound 50 was toxic at 1 p.p.m.

Compounds 41, 44, and 50 varied in their toxicity to goldfish, and only compound 50 killed all specimens at 10 p.p.m. None was toxic to the species at 1 p.p.m. Compound 58 was nontoxic at either concentration.

2',6'-diethyl-3,5-dinitrobenzanilide (compound 71, table 8) was nontoxic to rainbow trout and goldfish but caused sounding reaction among the rainbows at 0.1 and 1 p.p.m.

DISCUSSION

Chemical structures which exhibit the greater toxicity to rainbow trout and goldfish were nitrosalicylanilides. We attribute this to the ortho (2) hydroxy substitution on the acid moiety. Baichwal et al. (1960a) and Starkey and Howell (1965) made similar findings with respect to fungi and sea lampreys respectively. In contrast, benzanilides which do not contain the hydroxy substitution in the ortho position are relatively nontoxic to the test fishes.

The ortho (2) hydroxy substitution also appears to accelerate the biological activity of a compound, because the rainbow trout and goldfish often succumbed in tests within 3 hours.

The substitution of meta (3') nitro on the benzoic acid moiety of benzanilides produced greater toxicity to rainbow trout and goldfish than other nitro substitutions. A nitro substitution in the para (4) position had the least effect of any nitro substitution.

Substitutions of halogens in the para (4') position on the aniline moiety of salicylanilides markedly increased toxicities to fish. They also increase the speed of toxic action. Furthermore, the size of halogen atom has a dramatic effect on selective activity between rainbow trout and goldfish. A para substitution of iodine (atomic weight 127) is most toxic to rainbow trout, whereas bromine (atomic weight 80) is equally toxic to the two species. Chlorine (atomic weight 35) in the para position, however, is selectively toxic to goldfish.

Of the halogen substitutions, those in the ortho (2') position on the aniline moiety of either benzanilides or salicylanilides had least effect. One noteworthy exception occurred when an additional substitution of a nitro was made in the para (4') position. The result was greater toxicity to goldfish over rainbow trout. Similarly, the chloro substitution in the para (4') position in 4'-chloro-3-nitrosalicylanilide caused selective toxicity to goldfish. This particular change in the structural configuration strengthens the concept that selective toxicity occurs when the molecular geometry of a toxicant fits the receptor geometry of the fish. Baichwal et al. (1960b) investigated the fungicidal properties of salicylanilides and derivatives and indicated that activity may be attributed to interference to an enzyme or enzyme system(s) by hydrogen bonding or chelation via the phenol (ortho (4') hydroxy-) and amidic oxygen atoms. Furthermore, according to Ariëns (1964), the introduction of an electron spending group, such as the hydroxy (-OH) in the ortho or para position, increases the electron density in a conjugated system. Conversely, the nitro group decreases the electron density of this system.

Our further substitution of a bromine in the sub-meta (5) position on the salicylic acid moiety of the para (4') halo-3-nitrosalicylanilides gave even greater toxic action to fish in general. Baichwal et al. (1960a) noted a similar increase in toxicity by 5-chloro substitutions on fungicides.

CONCLUSIONS

The chemical structure of salicylanilides and benzanilides was related to toxicity and selectivity to rainbow trout and goldfish. Salicylanilides were more toxic than benzanilides to the fishes. The ortho (2) hydroxy substitution of salicylanilide accelerates biological activity against fish. The meta (3) nitro substitution on the salicylanilides and benzanilides increases toxicity to fish. The para (4') halogen substitution on nitrosalicylanilides markedly increased toxicities to fish and enhanced the speed of toxic action. The size of the halogen atom substituted on 3-nitrosalicylanilide affected selective toxicity between rainbow trout and goldfish. The ortho (2') halogen substitution on 3-nitrosalicylanilide became selectively toxic to goldfish by adding a para (4') nitro on the aniline moiety. The para (4') halogen substitution on 5-nitrosalicylanilides caused slight selective toxicity to goldfish. A para (4') chloro substitution on the 2',5'-dimethoxy-3-nitrosalicylanilide resulted in selective toxicity to goldfish. A bromine substituted in the sub-meta (5) position on para (4') halo-3-nitrosalicylanilides increased toxicity to fish.

SUMMARY

Relations between the chemical structures of salicylanilides and benzanilides and their toxicity to rainbow trout and goldfish were investigated. Single and multiple substitutions of alkyl, nitro, and halo groups on the salicylanilides and benzanilides were tested in standard, static bioassays with fingerling fish at the Fish Control Laboratory.

The nitrosalicylanilides were more toxic to the fish than benzanilides. A hydroxy substitution at ortho (2) appears to accelerate toxic action. The nitro substitution at meta (3) or sub-meta (5) on the benzoic acid moiety caused greater toxicity to the fish than other nitro substitutions.

Halogens in the para (4') position on the aniline moiety of salicylanilides markedly increased toxicities to fish and accelerated toxic action. The size of the halogen atom was found to have a great effect on selective activity between the two fishes. Iodine in the para (4') position produced greater toxicity to rainbow trout, whereas chlorine in the same position brought about an increase in toxicity to goldfish.

Halogens in the meta position were less toxic than para, and least toxic in the ortho position. An exception occurs when the chloro in the ortho (2') position was accompanied by a nitro in the para (4') position. The resulting compound was selectively toxic to goldfish. Halogenation in the sub-meta (5) position on the salicylic acid moiety produced even greater toxicity to both species.

The results of changes in structural configuration of salicylanilides support the concept that general and selective toxicity occurs when molecular geometry of a toxicant conforms to the receptor geometry of the fish.

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10. Evaluation of p,p′-DDT as a Reference Toxicant in Bioassays

By Leif L. Marking, Chemist
Fish Control Laboratory
Bureau of Sport Fisheries and Wildlife
La Crosse, Wisconsin

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EVALUATION OF p,p'-DDT AS A REFERENCE TOXICANT IN BIOASSAYS

By Leif L. Marking, Chemist
Fish Control Laboratory, La Crosse, Wis.

ABSTRACT.--p,p'-DDT was tested as a reference standard toxicant against 19 species of freshwater fish, including 39 lots from 10 sources. In particular, the rapidity, nonselectivity, and consistency of its toxicity to fish were evaluated in 96-hour static bioassays. The chemical was rapidly and consistently toxic to lake trout, carp, green sunfish, bluegill, and yellow perch. It lacked either rapid or consistent toxicity to rainbow trout, brook trout, goldfish, fathead minnows, and longear sunfish in 96-hour tests. Thus, p,p'-DDT is of limited usefulness as a reference standard toxicant in large bioassays with many species of fish.

Necessity for standardization of facilities and techniques in bioassay, and reproducibility and comparability of results from test to test and investigation to investigation was recognized early in the fish control program. Others have also emphasized this necessity. Hart, Doudoroff, and Greenbank (1945) stated the need for a reference evaluation in bioassays to serve as a link between work of different investigators and to compare the relative toxicities of substances. Warren and Doudoroff (1958) observed that living organisms are highly variable and in order to obtain reproducible results in bioassays, uniformly tolerant test organisms must be found. Cutting et al. (1959) pointed out the unique advantages of fish as indicator organisms, but noted the persistent difficulties in making needed comparisons of bioassays because of the variety of fishes used. Cope (1961) suggested that it is essential to have some measure of the condition of test fish which are used in bioassays. Douglas and Irwin (1962) stressed the fact that results of independent bioassays often cannot be related because the comparative resistance of many test fishes has not been established. They pointed out the need for knowledge on the reactions of different species of fish when exposed to a particular toxicant.

Standardization of bioassay facilities and methods at the Fish Control Laboratories, La Crosse, Wis., and Warm Springs, Ga., were reported by Lennon and Walker (1964). The reproducibility and comparability of results in bioassays may be achieved best by employing a standardized test fish, but there is none at present. In lieu of a standard test fish, the potentials of p,p'-DDT as a standard reference chemical in bioassays were investigated.

A standard reference chemical should have the following qualifications:

1. A rapid, nonselective, and consistent toxicity to fish.
2. Uncommon in nature, thus reducing risks of previous exposure among fish.
3. A known mode of action on fish.

The purpose of this study was to determine whether p,p'-DDT has the first qualification. Accordingly, we evaluated the toxicity of the chemical against 19 species of freshwater fish, including 39 separate lots of fish from 10 different sources.

METHODS AND MATERIALS

The bioassays of p,p'-DDT [1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethanol] at La Crosse and Warm Springs were static tests

in 5-gallon glass jars as described by Lennon and Walker (1964). Stock solutions of the reagent grade chemical were made in acetone, and aliquots were added directly to bioassay vessels. The quantity of acetone in bioassays never exceeded 1 ml. per 1. The 96-hour LC_{50} values (concentrations producing 50-percent mortality) were calculated according to the method of Litchfield and Wilcoxon (1949). This method gives a 95-percent confidence interval, the range in which 50 percent of the fish die 95 percent of the time.

The species and sources of fish are listed in table 1. Most of the lots were received from National Fish Hatcheries, but one lot of brook trout was received from a Wisconsin State Hatchery and two species were collected at the Necedah National Wildlife Refuge in Wisconsin. A "lot" is a particular group of fish of a species received from a hatchery in a single shipment.

TABLE 1.--Species and source of fish used in bioassays of p,p'-DDT

Common name	Technical name	Source
Rainbow trout.....	Salmo gairdneri	Manchester NFH, Ia.
Brown trout.....	Salmo trutta	Manchester NFH, Ia.
Brook trout.....	Salvelinus fontinalis	Lake Mills NFH, Wis.
[illegible].....	Salvelinus fontinalis	St. Croix Falls SFH, Wis.
Lake trout.....	Salvelinus namaycush	Jordan River NFH, Mich.
Northern pike.....	Esox lucius	Yankton NFH, S.D.
[illegible].....	Carassius auratus	Lake Mills NFH, Wis.
Northern redbelly dace.....	Chrosomus eos	Necedah NWR, Wis.
[illegible].....	Cyprinus carpio	Lake Mills NFH, Wis.
[illegible].....	Pimephales promelas	Lake Mills NFH, Wis.
Black bullhead.....	Ictalurus melas	Guttenberg NFH, Ia.
Channel catfish....	Ictalurus punctatus	Fairport NFH, Ia.
Brook stickleback..	Eucalia inconstans	Necedah NWR, Wis.
Green sunfish.....	Lepomis cyanellus	Lake Mills NFH, Wis.
Pumpkinseed.....	Lepomis gibbosus	Lake Mills NFH, Wis.
[illegible].....	Lepomis macrochirus	Lake Mills NFH, Wis.
Bluegill.....	Lepomis macrochirus	Warm Springs NFH, Ga.
Longear sunfish....	Lepomis megalotis	Fairport NFH, Ia.
Largemouth bass....	Micropterus salmoides	Genoa NFH, Wis.
Yellow perch.....	Perca flavescens	Lake Mills NFH, Wis.
Fresh water drum...	Aplodinotus grunniens	Lake Mills NFH, Wis.

Upon delivery, the fish were maintained in a fish holding house and fed commercial pellets. The lengths and weights of representative samples in each lot were measured routinely. The fish at La Crosse and Warm Springs were held and tested at 12° and 17° C., respectively. Before testing, each lot was in quarantine for 10 days; food was withheld the last 3 days. If, after quarantine, a lot of fish was judged acceptable for bioassay, it was used in tests of p,p'-DDT and candidate fish controls. The tests with p,p'-DDT were repeated biweekly for as long as a lot remained on hand in usable condition. Subsequent acquisitions of the same species were assigned different lot numbers even though they may have come from the same hatchery stock as a previous lot.

Mortalities of the test fish were recorded throughout each 96-hour bioassay. Preliminary investigations disclosed that p,p'-DDT produces similar effects on all species of fish, although there are slight variations in the behavior of different species. Reactions in catfishes, for instance, are less obvious than in trout. Generally, irritation and excitation are readily noticeable in the earlier stages of response. Swimming becomes erratic, upside down, on a side, with surfacing and colliding with walls of the container. The swimming is continuous until the final stages of response. Total loss of equilibrium, loss of reflex reactivity, and cessation of locomotion immediately precede death. Moribund fish often turn light in color. The operculums are usually distended and the spine is sometimes curved, indicating convulsive reactions at the time of death.

RESULTS

The toxicity of p,p'-DDT differed greatly among the 19 species of fish. The compound was consistently very toxic to some fishes but less and inconsistently so to others (table 2). The toxicity also varied among lots of a species, and details are given in tables 3 to 7.

TABLE 2.--Order of toxicity of p,p'-DDT to various species of fish

Species	Number of lots	Number of tests	Mean LC_{50} in p.p.b.
Largemouth bass.....	1	1	0.8
Yellow perch.....	2	3	0.9
Northern pike.....	1	1	1.7
Green sunfish.....	3	8	4.5
Pumpkinseed.....	2	5	4.5
Bluegill.....	8	10	4.5
Carp.....	4	6	8.2
Longear sunfish.....	1	2	8.7
Lake trout.....	1	2	9.3
Freshwater drum.....	1	1	10.0
Rainbow trout.....	4	8	10.7
Brown trout.....	1	1	10.9
Brook trout.....	2	4	11.5
[illegible].....	[illegible]	[illegible]	[illegible]
Black bullhead.....	1	4	25.8
Goldfish.....	4	7	58.7
Brook stickleback.....	1	1	67.0
Northern redbelly dace.....	1	1	68.0

TABLE 3.--Toxicity of p,p'-DDT to trout and northern pike in 96 hours at 12°C.

Species and lot	Average weight (grams)	Date tested	LC_{50} (p.p.b.)	95-percent confidence interval
Rainbow trout:				
Lot 16a	0.9	3-24-64	14.0	12.8-15.4
Lot 17	0.9	4-10-64	4.6	2.9- 7.4
Do.	1.2	4-24-64	7.2	6.0- 8.7
Lot 27	1.6	5-15-64	15.0	12.0-19.0
Do.	1.9	5-22-64	17.0	14.4-20.1
Do.	2.5	6- 5-64	13.0	9.7-17.4
Do.	3.1	6-19-64	12.0	10.7-13.4
Lot 159	0.5	2- 5-65	2.4	1.7- 3.3
Brown trout: Lot 26	4.0	6- 5-64	10.9	9.6-12.3
Brook trout:				
Lot 18	1.4	4-10-64	7.2	5.2-10.1
Do.	3.3	4-24-64	17.0	14.3-20.2
Do.	3.3	5- 1-64	20.0	11.9-33.6
Lot 161	0.4	2- 5-65	1.8	1.2- 2.4
Lake trout:				
Lot 78	2.5	8-21-64	9.1	8.0-10.4
Do.	2.8	9-18-64	9.5	8.5-10.6
Northern pike: Lot 34	0.5	5-30-64	1.7	1.4- 2.0

TABLE 4.--Toxicity of p,p'-DDT to cyprinids in 96 hours at 12°C.

Species and lot	Average weight (grams)	Date tested	LC_{50} (p.p.b.)	95-percent confidence interval
Goldfish:				
Lot 1	2.5	3-4-64	76.0	27.0-213.0
Lot 19	2.4	3-31-64	27.0	15.0- 50.0
Do.	2.4	4- 3-64	32.0	17.0- 61.0
Lot 75	1.9	8-11-64	180.0	125.9-257.4
Lot 119	0.8	11-13-64	40.0	22.8- 70.0
Do.	0.7	11-27-64	35.0	26.9- 47.2
Do.	1.0	12-11-64	21.0	13.1- 33.7
Northern redbelly dace:				
Lot 154	1.1	1- 8-65	68.0	41.2-112.2
Carp:				
[illegible]	2.2	9- 4-64	9.2	4.6- 18.4
[illegible]	2.0	9-11-64	4.0	1.3- 12.0
[illegible]	2.1	9-25-64	11.3	8.7- 14.7
[illegible]	2.5	10- 9-64	12.0	6.0- 24.0
[illegible]	0.8	1- 8-65	6.9	5.1- 9.3
[illegible]	0.6	1-22-65	6.0	4.7- 7.6

TABLE 5.--The toxicity of p,p'-DDT to catfishes and brook stickleback in 96 hours at 12°C.

Species and lot	Average weight (grams)	Date tested	LC_{50} (p.p.b.)	95-percent confidence interval
Black bullhead:				
[illegible]	2.1	10- 9-64	42.0	33.6-52.5
[illegible]	2.4	10-30-64	23.5	16.1-34.3
[illegible]	2.3	11-13-64	17.0	12.6-23.0
[illegible]	2.2	11-27-64	20.0	13.3-30.0
[illegible]:				
[illegible]	1.9	9- 4-64	17.5	8.3-36.8
[illegible]	2.0	9-18-64	17.5	10.3-29.8
Brook stickleback:				
Lot 127	1.6	12- 4-64	67.0	54.6-82.4

TABLE 6.--Toxicity of p,p'-DDT to centrarchids in 96 hours at 1[illegible]

Species and lot	Average weight (grams)	Date tested	LC_{50} (p.p.b.)	95-percent confidence interval
Green sunfish:				
Lot 25	0.7	5-22-64	2.8	[illegible]
[illegible]	[illegible]	[illegible]	3.0	[illegible]
[illegible]	[illegible]	8-20-64	3.9	[illegible]
[illegible]	[illegible]	10- 9-64	6.7	[illegible]
[illegible]	[illegible]	[illegible]	1.4	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	0.8	12-31-64	3.6	[illegible]
[illegible]	0.8	1-22-65	5.0	[illegible]
[illegible]				
Lot 118	1.4	10-29-64	7.5	[illegible]
[illegible]	1.4	11-13-64	6	[illegible]
[illegible]	1.3	12-11-64	[illegible]	[illegible]
[illegible]	1.3	12-31-64	[illegible]	[illegible]
Do.	1.3	1- 8-65	[illegible]	[illegible]
[illegible]				
[illegible]	0.6	4-24-64	4.3	[illegible]
[illegible]	0.6	5- 8-64	1.7	[illegible]
[illegible]	1.1	9-18-64	3.0	[illegible]
[illegible]	1.3	10-16-64	7.0	[illegible]
[illegible]	0.6	11-29-64	7.0	[illegible]
[illegible]	1.1	12- 4-64	3.6	[illegible]
[illegible]	0.8	1- 5-65	1.2	[illegible]
[illegible]	[illegible]	12- 3-64	4.6	[illegible]
[illegible]	1.2	12-14-64	9.4	[illegible]
[illegible]	1.0	12-28-64	2.8	[illegible]
Longear sunfish:				
Lot 124a	1.0	11-20-64	4.9	4.0- 6.0
Do.	1.0	12- 4-64	12.5	7.6-20.6
Largemouth bass: Lot 57	0.5	7-17-64	0.8	0.7- 0.9

TABLE 7.--Toxicity of p,p'-DDT to yellow perch and freshwater drum in 96 hours at 12°C.

Species and lot	Average weight (grams)	Date tested	LC_{50} (p.p.b.)	95-percent confidence interval
Yellow perch:				
Lot 116	1.1	11-13-64	0.8	0.6- 1.0
Do.	0.8	11-27-64	0.6	0.4- 0.8
Lot 151	1.2	1-22-65	1.5	1.1- 2.0
Freshwater drum: Lot 117	3.3	10-30-64	10.0	5.6-18.0

The p,p'-DDT was most toxic to largemouth bass, yellow perch, and northern pike, in that order; mean LC_{50} values were 0.8, 0.9, and 1.7 p.p.b. The fish were small fingerlings, however, and they did not appear to hold up well in the 2 to 4 days of starvation during the test period. Had live food been available in quantity before and during the tests, the fish might have shown greater resistance to the toxicant. Furthermore, the holding and test temperature of 12° C. may have been below optimum for fingerlings of these species.

The mean LC_{50} of the toxicant was 4.5 p.p.b. for green sunfish, pumpkinseed, and bluegill (table 2). The toxicity ranged from 2.8 to 6.7 p.p.b. for green sunfish, 1.8 to 7.5 p.p.b. for pumpkinseed, and 1.2 to 9.4 p.p.b. for

bluegill, and the higher values for them occurred in October (table 6). Green sunfish in lot 25 doubled their average weight, 0.7 to 1.4 g. in a month, and the LC_{50} increased from 2.8 to 3.9 p.p.b. In contrast, green sunfish in lot 95 lost in average weight from 1.1 to 0.7 g. and LC_{50} from 6.7 to 4.4 p.p.b. The LC_{50} values for lots of the three species which merely maintained their average weights over 2-week to 1-month periods varied up and down, but the trend was generally downward.

The p,p'-DDT produced consistent results among bluegills tested at La Crosse and Warm Springs. The biweekly LC_{50} values for one lot of longear sunfish increased from 4.9 to 12.5 p.p.b. Since the confidence intervals did not overlap, the difference was significant (table 6).

The chemical was less toxic to carp than to most of the centrarchids but more toxic than to trouts (table 2). It was tested three times against carp in lot 83 which fed, grew farily rapidly, and became more resistant.

The toxicity of the compound was intermediate to trouts (table 2). Of the four species, it was most toxic to lake trout and least to brook trout. The data suggest selectivity to some degree among these closely related species. Hatch (1957) related an interesting example of different responses among salmonids to DDT. An aerial application of the insecticide to a hatchery watershed was sublethal to rainbow and brook trout but killed all landlocked Atlantic salmon. His further observations confirmed that the trouts could survive concentrations deadly to the salmon.

The bioassays included four lots of rainbow trout, and the LC_{50} values for them were inconsistent. Generally, the larger fish were more resistant to the chemical. The mean LC_{50} for trout over 1.5 g. was approximately 14. p.p.b. while the value dropped to 7 p.p.b. for fish less than this weight.

The candidate chemical was less toxic to channel catfish and black bullhead (table 2). There was only one lot of channel catfish available, however, and the fish showed identical tolerances in tests 2 weeks apart. The LC_{50} for black bullhead declined from 42 to 17 p.p.b. over a 7-week period (table 5).

The p,p'-DDT was one-fifth as toxic to goldfish as to trouts. Moreover, the interlot and intralot differences in responses to the toxicant were great. The LC_{50} values for lots ranged from 21 to 180 p.p.b., and confidence intervals were extremely wide (table 4). These intervals do not overlap in all instances and suggest that the tolerance differs.

The p,p'-DDT was least toxic to brook stickleback (table 5) and northern redbelly dace (table 4), and their resistances exceeded the mean tolerance of goldfish. The LC_{50} values are six times as great as the mean for trouts (table 2).

Tests of the toxicant against fathead minnows presented unique problems. The results were neither rapid nor consistent. The toxicity did not increase uniformly with increased concentrations up to 1,000 p.p.b. within 96 hours. A reliable LC_{50} could not be obtained in repeated trials. Furthermore, a marked abdominal distention occurred in exposed fish. It began at 24 hours and became more pronounced at 96 hours. Examination of the peritoneal cavity revealed an enlarged air bladder. It is not known at present what relation this distention has to the toxicant. Henderson, Pickering, and Tarzwell (1959) noted the phenomenon among fathead minnows which had been exposed to chlorinated hydrocarbons. They indicated, however, that the LC_{50} of p,p'-DDT to fatheads was 32 p.p.b. in 24 hours and 26 p.p.b. in 96 hours. We could not confirm their results.

DISCUSSION

The p,p'-DDT was rapidly and consistently toxic to lake trout, carp, channel catfish, green sunfish, bluegill and yellow perch, but not so to rainbow trout, brook trout, and black bullhead. Results were unsatisfactory with goldfish and fathead minnows in 96-hour bioassays. The regression curves, plotted on logarithmic coordinates, in figure 1 illustrate the consistency of toxic responses in rainbow trout and goldfish. The calculated slope function

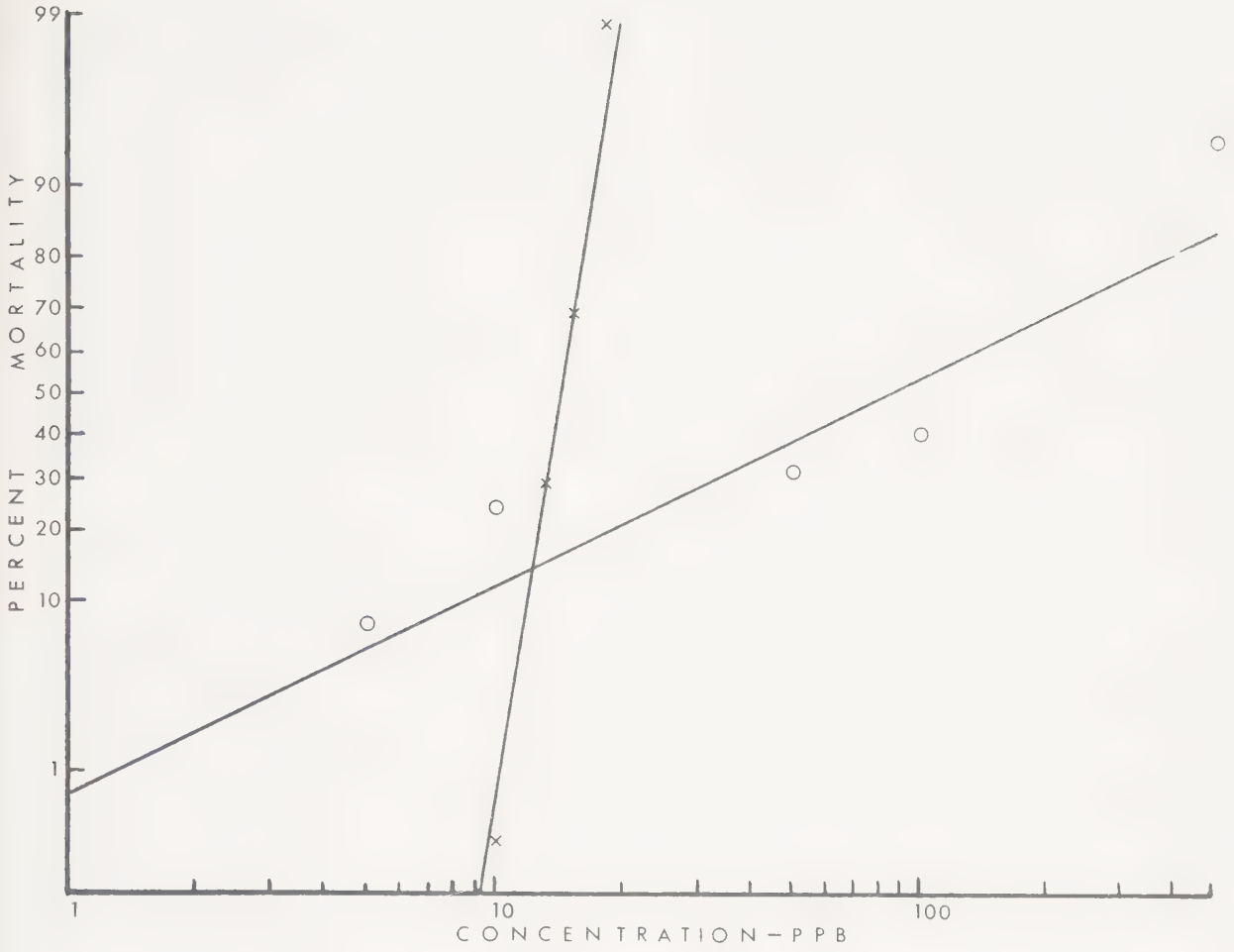

Figure 1.--Responses of rainbow trout (x) and goldfish (o) to p, p'-DDT.

for rainbow trout is 1.16 whereas that for goldfish is 6.02.

The regression curve for rainbow trout (lot 16a) indicates toxicity at very low levels. The slope of the curve reveals a narrow margin between survival and mortality. The confidence intervals are relatively narrow, thus indicating that toxicity is more accurately defined.

The regression curve for goldfish (lot 1) contrasts sharply with that for rainbow trout. The curve is flat rather than steep, and it shows that increased concentrations in the survival and mortality range produce little effect within a 96-hour bioassay. The confidence intervals are wide, and the toxicity of p,p'-DDT to goldfish is not defined accurately.

The results with p,p'-DDT further demonstrated that lots of fish within a single species may vary significantly in responses. The variations may be due to several factors. Centrarchids appeared to have seasonal cycles in relative resistance to the toxicant. Green sunfish, pumpkinseed, and bluegill displayed higher tolerances in autumn (table 6). It was impossible, however, to evaluate total cyclic effects since the fish were not available for more than a few months of the year. Shell (1961) found systematic and seasonal variations in blood components of smallmouth bass. He noted that most of the changes took place in August and suggested that changes in the anabolic-catabolic balance were responsible for the cyclic concentrations of the various blood components. This may explain why fish are at times more vulnerable to toxicants or other stresses.

Holden (1962) determined that fish in poor condition or with a low reserve of fatty tissue are more sensitive to DDT. This was noticeable in some of our tests with pumpkinseed, bluegill, and yellow perch. In contrast, relatively healthy fish which were feeding and gaining in weight tended to become more resistant to the toxicant. Examples of increasing resistance were afforded by rainbow trout (lot 17), brook trout (lot 18), goldfish (lot 19), carp (lot 83), and green sunfish (lot 25). Phillips, Livingston, and Dumas (1960) reported that starved brook trout have a tendency to increase in water content and decrease in body fat and protein. Such changes in body chemistry may contribute to decreased resistances of the fish to the reference compound.

The bioassays of p,p'-DDT showed that fish obtained from different sources display different tolerances. Geographic location, water quality, pond fertilization, herbicide applications, and feeding and handling during the rearing period probably influence the relative tolerance of specimens to a toxicant. Lots of brook trout from Lake Mills National Fish Hatchery and St. Croix Falls State Fish Hatchery varied considerably in resistance. On the other hand, the bluegills from Lake Mills and Warm Springs National Fish Hatcheries reacted quite similarly.

A good reference standard chemical should be sufficiently uncommon in nature that there is little risk that bioassay fishes have been exposed to it or its closely related compounds. The p,p'-DDT hardly meets this qualification, since bioassay fishes from various hatcheries may have been exposed previously to DDT. It is very widely used as a pesticide and resists decomposition. A hatchery in an agricultural watershed, for example, may very well be contaminated to some extent. Also, the natural or prepared diets of the fish are likely to contain small quantities of DDT. Such exposures to the insecticide may have influenced the tolerances of fish in our tests. Many of the trials were unsuccessful because all specimens either lived or died at concentrations of p,p'-DDT which had previously resulted in partial kills.

Cope (1960) reported that trout and whitefish retained DDT in considerable quantities almost a year after an aerial application for spruce budworm in a western watershed. King (1962) found that guppies became more tolerant to DDT in repeated exposures. Vinson, Boyd, and Ferguson (1963) observed that mosquitofish (Gambusia affinis) preexposed to DDT near treated cotton fields were considerably more tolerant than fish from untreated areas. Boyd and Ferguson (1964) observed that tolerance to DDT persisted through one to three generations of mosquitofish which were removed from an environment contaminated by the insecticide.

CONCLUSIONS

The p,p'-DDT partially fulfilled the qualification that a reference standard toxicant be rapidly, nonselectively, and consistently toxic. It was rapidly and consistently toxic to lake trout, carp, channel catfish, green sunfish, bluegill, and yellow perch. It was sufficiently toxic to brown trout, northern pike, northern redbelly dace, black bullhead, brook stickleback, largemouth bass, and freshwater drum, but there were not enough lots of each species to determine the consistence of toxicity. It lacked either rapid toxicity or consistent toxicity to rainbow trout, brook trout, goldfish, fathead minnows, and longear sunfish in 96-hour bioassays. The p,p'-DDT exhibited a selective toxicity among the 19 fishes instead of the desired nonselectivity. For example, the mean LC_{50} for largemouth bass was 0.8 p.p.b. whereas the LC_{50} for northern redbelly dace was 68 p.p.b. Moreover, a reliable LC_{50} for fathead minnows could not be obtained within a 96-hour bioassay.

There were intraspecific as well as interspecific differences in the sensitivity of fish to the toxicant. For example, the range of LC_{50} values for four lots of goldfish was 21 to 180 p.p.b. The p,p'-DDT was generally more toxic to small fish than large ones within a species. Thus, p,p'-DDT is limited in usefulness as a reference standard in large-scale bioassays with many species of freshwater fish. The tests against 19 species demonstrate the continuing need for an adequate

standard reference compound and/or a standard reference fish. In spite of the limitations, it is useful in the selection, testing, and evaluation of other candidate reference compounds.

SUMMARY

A standard reference compound is needed in the fish control program to facilitate reproducibility and comparisons in bioassays in which many species, sizes, conditions, and sources of fish, and many kinds and concentrations of chemicals are involved. The p-p'-DDT was tested for rapid, nonselective, and consistent toxicity against 19 fishes, including different lots of the same species and repeated samples within lots. The trials were accomplished in static bioassays in the laboratory at 12° C. We established the LC_{50} of p,p'-DDT for each lot of fish received from hatcheries or the field, but only after 10 days of quarantine in a fish-holding house. The tests were repeated biweekly for as long as a lot remained on hand in testworthy condition.

The p,p'-DDT was rapidly and consistently toxic in 96-hour bioassays to 6 of the 19 species. It was sufficiently toxic to 8 others, but there were insufficient lots to evaluate consistency. It lacked either rapid toxicity or consistent toxicity to five species. The mean LC_{50} for largemouth bass was 0.8 p.p.b., and the mean for northern redbelly dace was 68 p.p.b., thus it was more selective in its action than would be desirable.

There were intraspecific as well as interspecific differences in sensitivity among the fish to the toxicant. The chemical was more toxic to small fish than large ones within a species.

Since the toxicity of p,p'-DDT varied in rapidity, selectivity, and consistency against 19 species of freshwater fish, the compound is of limited usefulness as a standard reference in large-scale bioassays. The results are helpful, however, in the selection, testing, and evaluation of other candidate reference compounds.

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11. Evaluation of an Electronic Method of Measuring Hematocrits of Fish

By Richard A. Schoettger and Arnold M. Julin, Fishery Biologists
Fish Control Laboratory
Bureau of Sport Fisheries and Wildlife
La Crosse, Wisconsin

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EVALUATION OF AN ELECTRONIC METHOD OF MEASURING HEMATOCRITS OF FISH

By Richard A. Schoettger and Arnold M. Julin, Fishery Biologists
Fish Control Laboratory, La Crosse, Wis.

ABSTRACT.--Conductivity measurements of mammalian blood are considered satisfactory estimates of hematocrit. The YSI Electronic Hematocrit gives rapid and reproducible results with fish blood, and the readings can be corrected to microhematocrit values. In the low hematocrit range, however, readings do not change proportionately with microhematocrits; they are difficult to correct; but they may prove useful as a gross, rapid method for detecting anemia. Electrolyte and protein concentrations in fish blood influence the electronic hematorcrit; the relative size of erythrocytes may also.

Hematological parameters are used frequently to estimate the physiological condition of fish. Among them, the hematocrit is one of the easier values to secure. Procedures, problems, and modifications associated with microhematocrit determinations in fish were discussed by Hesser (1960), Snieszko (1960), Larsen and Snieszko (1961), and Mairs and Kennedy (1962).

The microhematocrit, or centrifuge, technique is better suited to laboratory routine than to measurements of hematocrit in the field. Electric line power is needed for the centrifuge, and transportation of blood samples to the laboratory is time consuming. There is need for a method which complements the centrifuge technique in the laboratory and is convenient for studies in the field.

An electrical device for relating the conductivity of mammalian blood to the hematocrit was described by Okada and Schwan (1960). The pointed out that its validity is affected by changes in serum conductivity or the average size of erythrocytes. Kernen et al. (1961) investigated the reproducibility of results obtained with the instrument on human blood and studied the effect of anticoagulants and blood protein levels on conductivity. Further tests were conducted by the Yellow Springs Instrument Company, which manufactures a similar instrument called an Electronic Hematocrit.

Our investigation was designed to study the comparability of the YSI electronic and centrifuge methods for measuring hematocrits in fish, the reproducibility of the electronic hematocrits, and some physiological variables in the blood of fish which could influence conductivity.

METHODS AND MATERIALS

Hematocrits were determined as described by Larsen and Snieszko (1961) for the centrifuge method and according to the manufacturer's instructions for the electronic method.

Briefly, the YSI Model 30 Electronic Hematocrit is portable, weighs 2.8 pounds, is powered by a 6-volt mercury cell, and is compensated for use at 50° to 105° F. A blood sample of at least 0.02 cc. is drawn and placed between two electrodes imbedded

in a glass cell. The cell is placed between the circuit contacts, a button is pressed, and the hematocrit is read directly from the instrument. The reproducibility of readings is reported by the manufacturer to be 0.1 unit with a standard deviation of ± 0.3 unit. Anticoagulants other than heparin and abnormal levels of serum proteins in human blood influence conductivity.

The operating principle of the electronic hematocrit is based on the insulating characteristics of the erythrocyte membrane which separates the conductive interior from the conductive serum outside the cell. The YSI instrument contains a bridge circuit. Two arms of the bridge consist of a precision, center-tapped transformer. The third is a thermistor-resistor which automatically compensates for ambient and glass cell temperatures. A fixed volume of blood within a glass cell constitutes the unknown fourth resistance. Unbalance of the bridge is a measure of the concentration of erythrocytes (manufacturer's specifications; and Okada and Schwan, 1960).

Blood samples were collected from fish by cardiac puncture with a syringe or from severed caudal vessels directly into capillary tubes. The syringes, tubes, and scalpels were coated with heparin.

The reproducibility of the two hematocrit methods was estimated by repeated measurements on relatively large samples of blood. The samples were secured from one each shovelnose sturgeon, spotted sucker, northern redhorse, brown bullhead, white bass, largemouth bass, and walleye. During the measurement of hematocrits a magnetic mixer was used to maintain uniform distribution of red blood cells within a sample, as described by Snieszko (1963).

Tests of the comparability of the hematocrit methods were conducted with fingerling and larger-size fish including: rainbow trout, brown trout, brook trout, lake trout, and goldfish. Two capillary tubes were filled from each specimen. One was spun in an International Microcapillary Centrifuge, Model MB, and the hematocrit determined with a plastic reader. The second sample was utilized for electrical measurements.

The effects of sodium chloride and protein concentrations on electronic hematocrits of rainbow trout and carp were determined in an experimental design similar to that of Kernen et al. (1961) except that the tests were conducted with salines containing 0.65-, 0.75-, 0.85-, and 1.0-percent sodium chloride. According to Wolf (1963) this range of salines has been used in physiological studies of fish. Powdered bovine albumin was added to the salines to obtain protein levels of 1 to 9 grams per 100 cc. This albumin was selected because it was used by Kernen et al. (1961). The erythrocytes were separated from heparinized blood by centrifuging at 850 r.p.m. for 15 minutes. The cells were washed twice in the appropriate saline at 20° C. and resuspended in various protein-saline solutions to give approximate hematocrits of 25 to 30 percent. A third saline rinse occasionally resulted in hemolysis of the erythrocytes in the final resuspension. Wolf (1959) found that red cells of salmonids coagulated in various sodium chloride solutions which did not contain anticoagulant. In our tests, residual heparin probably served a similar function. Ionic coagulants such as the oxalates alter the conductivity of samples (Kernen et al., 1961).

Throughout the tests, capillary tubes were examined for clotting and hemolysis. The electronic cells were checked for clots. It was essential to conduct electrical measurements rapidly to minimize the effect of erythrocyte sedimentation within the glass cell.

The trout and goldfish were obtained from State and National fish hatcheries. The other species were collected in the Mississippi River. They were maintained in flowing well water at the Fish Control Laboratory for at least 2 months before use.

RESULTS AND DISCUSSION

REPRODUCIBILITY OF HEMATOCRITS

The tests with seven species of fish to evaluate the relative reproducibility of the two hematocrit methods indicated that the centrifuge technique was somewhat superior (table 1). The standard deviations, for example, were smaller except in the case of the shovelnose sturgeon. In contrast, the electronic hematocrits were consistently low and standard deviations were greater. Statistical tests for each species of fish demonstrated a significant difference between the results of the two hematocrit methods at the 0.01 level.

TABLE 1.--Comparative reproducibility of electronic and centrifuge hematocrits

Species of fish	Hematocrit								Centrifuge minus electronic
	Electronic				Centrifuge				
	Number of hematocrits	Mean	Range	Standard deviation	Number of hematocrits	Mean	Range	Standard deviation	
Shovelnose sturgeon..	12	15.5	14.0-17.0	0.8	12	22.0	20.5-23.0	1.0	6.5
Spotted sucker.....	12	20.0	17.0-23.0	1.4	10	33.5	32.0-34.0	0.7	13.5
Northern redhorse....	20	31.0	28.5-32.5	1.2	24	33.0	31.0-34.5	0.7	2.0
Brown bullhead.....	12	16.0	14.0-18.0	0.9	12	22.0	21.0-22.5	0.5	6.0
White bass.....	12	14.0	12.0-18.0	1.5	11	31.0	30.0-32.5	0.7	17.0
Largemouth bass.....	8	14.5	12.0-16.0	1.4	12	26.0	24.0-27.0	1.0	11.5
Walleye.....	9	28.0	25.0-29.0	1.4	12	40.0	39.0-41.5	0.7	12.0

COMPARABILITY OF HEMATOCRITS

The electronic hematocrit was considered sufficiently reproducible. The differences between the means of the two methods suggested a need to correct electronic readings for individual and species variation. The regressions of electronic hematocrit on centrifuge hematocrit were determined for five species (fig. 1). The mean centrifuge values for the various species were as follows: rainbow trout, 40.4; brown trout, 33.0; brook trout, 37.1; lake trout, 35.7; and goldfish, 42.8. The mean electronic hematocrits, depending on species, were generally 20 to 60 percent lower than the means of the centrifuge hematocrits. Statistical tests showed that the differences between the means of the two methods were significant at the 0.01 level.

The electronic hematocrit can be corrected to compare with centrifuge value by using the regressions in figure 1 or correcting with mean differences between the methods (table 2). The second readings in the table will be discussed later.

The regression technique is preferable because differences between hematocrit methods vary with the magnitude of the hematocrit. For example, an electronic hematocrit of 10 for rainbow trout corresponds to a centrifuge value of 25 and a reading of 30 to 49.5 when corrected by the regression. Using the mean difference as a correction, these values would be 27.5 and 47.5.

The regressions and the differences between means for rainbow and brown trout are similar (fig. 1, table 2). The 95-percent confidence intervals indicate that the same factor could be used to correct the electronic hematocrits of either salmonid. Separate corrections are required for other species.

The standard deviations of the differences between electronic and centrifuge hematocrits indicate a considerable variation about their means (table 2). Since the electronic hematocrit is relatively reproducible, and the measurements were made on the same

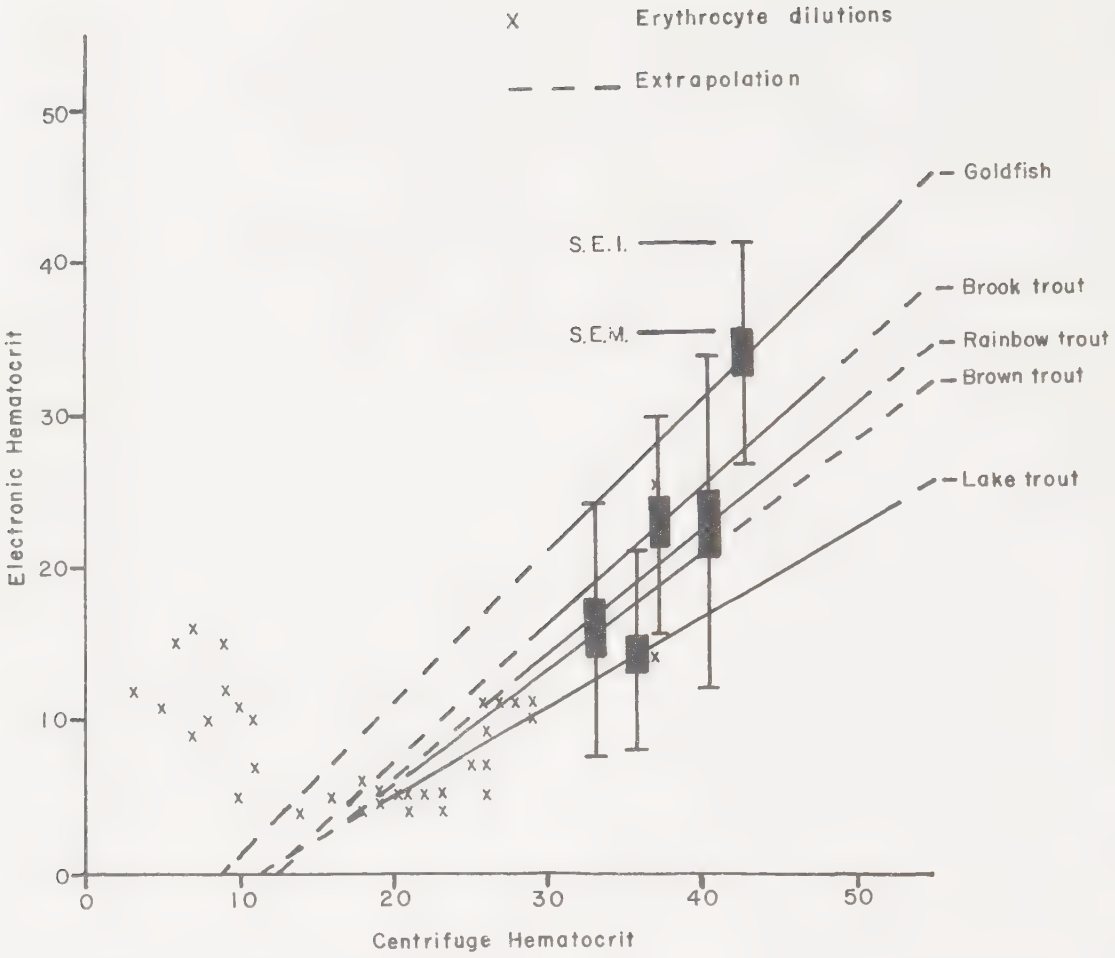

Figure 1.--Plot of electronic hematocrit against centrifuge hematocrit. Straight-line regression equations were computed for the data. S.E.I. and S.E.M. correspond to standard errors of individual and mean estimates respectively.

TABLE 2.--Mean differences between hematocrit methods for five species of fish

Species and electronic reading	Number of measurements	Mean difference; centrifuge minus electronic	Range of differences	Standard deviation	Standard error	95-percent confidence interval
Rainbow trout:						
t reading	27	17.4	9-26	5.0	0.97	15.4-19.4
cond reading	17	10.0	4-17	3.4	0.82	8.3-11.7
Brown trout:						
First reading	22	17.3	9-24	4.0	0.84	15.6-19.1
	2	8.3	2-15	3.7	0.80	6.6- 9.7
	23	14.3	5-19	3.3	0.70	12.9-15.9
	23	6.3	1-12	3.1	0.65	5.0- 7.7
Lake trout:						
First reading	55	21.4	12-31	4.6	0.61	20.2-22.6
nd reading	55	10.9	3-17	3.2	0.43	10.0-11.8
Goldfish:						
irst reading	30	9.1	1-16	3.4	0.62	7.8-10.4
Second reading	19	1.4	[1] 4- 5	2.6	0.60	0.1- 2.6

[1] All centrifuge hematocrits exceeded electronic readings except for three values in this group. The range extended from four units above to five units elow the centrifuge hemat .

samples, the standard deviations suggest variations in sample conductivity due to factors other than percentage of cells. For this reason, the corrected electronic hematocrit should only be considered an estimate of the centrifuge hematocrit.

The extrapolations of regressions in figure 1 converge toward a common intercept as the hematocrit decreases. Unfortunately, anemic fish were unavailable to test the extrapolations, but plasma dilutions of rainbow erythrocytes were used to simulate anemia. The measurements of simulated samples were comparable to the predicted values at centrifuge hematocrits above 25; between 10 and 25, the readings ranged from 4 to 6 and increased with hematocrits less than 10. Thus the correction of electronic hematocrits of anemic fish may give misleading estimates of the centrifuge hematocrit. Readings of 4 to 6, however, might be used as gross, but rapid, indicators of anemia. Though severe anemia may give higher electronic values, the condition is usually detected by visual examination of the blood.

The conductivities of solutions containing 0.45- to 1.20-percent sodium chloride were tested on the YSI Electronic Hematocrit. A plot of the readings against the salt concentrations formed a curve similar to that shown in figure 1 for plasma dilutions of red blood cells. Salines of 0.60 to 0.75 percent sodium chloride gave electronic readins of 5 to 6. The similarity of the two curves suggests that the number of erythrocytes in blood affect the electronic hematocrit like changes in the electrolyte concentration. The electronic hematocrit of the plasma used to dilute the erythrocytes corresponded to that for a 0.86-percent solution of salt. A 0.86-percent saline has a freezing point similar to that for the bloods of most freshwater teleosts (Black, 1957). The data indicate that electronic measurements of plasma, or serum, might be used to estimate the electrolyte levels in fish blood.

Changes in the conductivity of a blood sample were observed upon re-reading. The value rose shortly after the first reading, increased to a peak, and then declined. The rate of change varied with individuals and groups of fish. The apex was usually attained in 3 to 7 minutes and occasionally approximated the value obtained by the centrifuge method. An examination of blood within the glass cell indicated that the change in conductivity may be related to sedimentation and an unequal distribution of erythrocytes between the electrodes. This factor may be significant when measurements are delayed or when several samples are collected before reading.

Limited studies were conducted on the possibility of using the second electronic reading to estimate hematocrit. The mean differences between hematocrit methods, using the second reading, are shown in table 2. A correction factor for the second reading, like the first, is needed to estimate hematocrit. The factors are considerably smaller and have lower standard deviations, and standard errors. The second reading is time consuming and requires the use of heparinized blood. We found that blood could be drawn directly into the glass cell, without heparinization, and the first reading made within approximately 30 seconds. The blood began to clot shortly afterward, and a second reading was not usually possible. The alternative for using the second electronic reading is the preliminary collection of blood in heparinized capillary tubes; however, the technique detracts from the ease and speed of the electronic hematocrit.

Although the electronic determination of hematocrit is relatively rapid, time is lost in cleaning the glass cells. Several rinses in distilled water and drying with acetone were usually adequate. If blood dried or clotted in the cell, it was difficult to remove. If it remained, it changed sample volumes.

EFFECTS OF SALINE AND PROTEIN ON ELECTRONIC HEMATOCRIT

Experiments were conducted with rainbow trout and carp to determine whether variations in the electrolyte and protein concentration of fish blood influence electronic hematocrit (table 3 and fig. 2). The electronic hematocrit was inversely proportional to the concentration

TABLE 3.--Effect of various protein and sodium chloride concentrations on the deviation of electronic from centrifuge hematocrits

Species and concentration of sodium chloride	Protein concentrations of (grams per 100 cc.)--					
	1	2	3	5	7	9
Rainbow trout:						
0.65 percent	-1.5	--	-0.5	+1.0	--	+2.0
Do	-1.0	--	0.0	+0.5	--	+1.5
[illegible] percent	--	--	-6.0	-2.5	--	-3.0
[illegible] percent	--	--	-7.0	-6.0	--	-4.0
[illegible] percent	-11.5	-12.0	-12.5	-9.5	-7.5	-5.0
[illegible]	-14.5	-14.0	-11.0	-9.0	-7.5	-8.0
[illegible] percent	-27.5	-24.5	-24.5	-22.0	-20.5	-18.0
[illegible]	-26.0	-24.5	-23.5	-21.5	-19.5	-19.0
Carp:						
[illegible] percent	0.0	--	+1.0	+2.5	--	+4.0
[illegible]	-1.0	--	+1.5	+0.5	--	+4.5
[illegible] percent	-14.0	-12.5	-11.5	-11.0	-7.5	-5.0
[illegible]	-15.5	-11.5	-12.5	-9.0	-7.5	-8.0

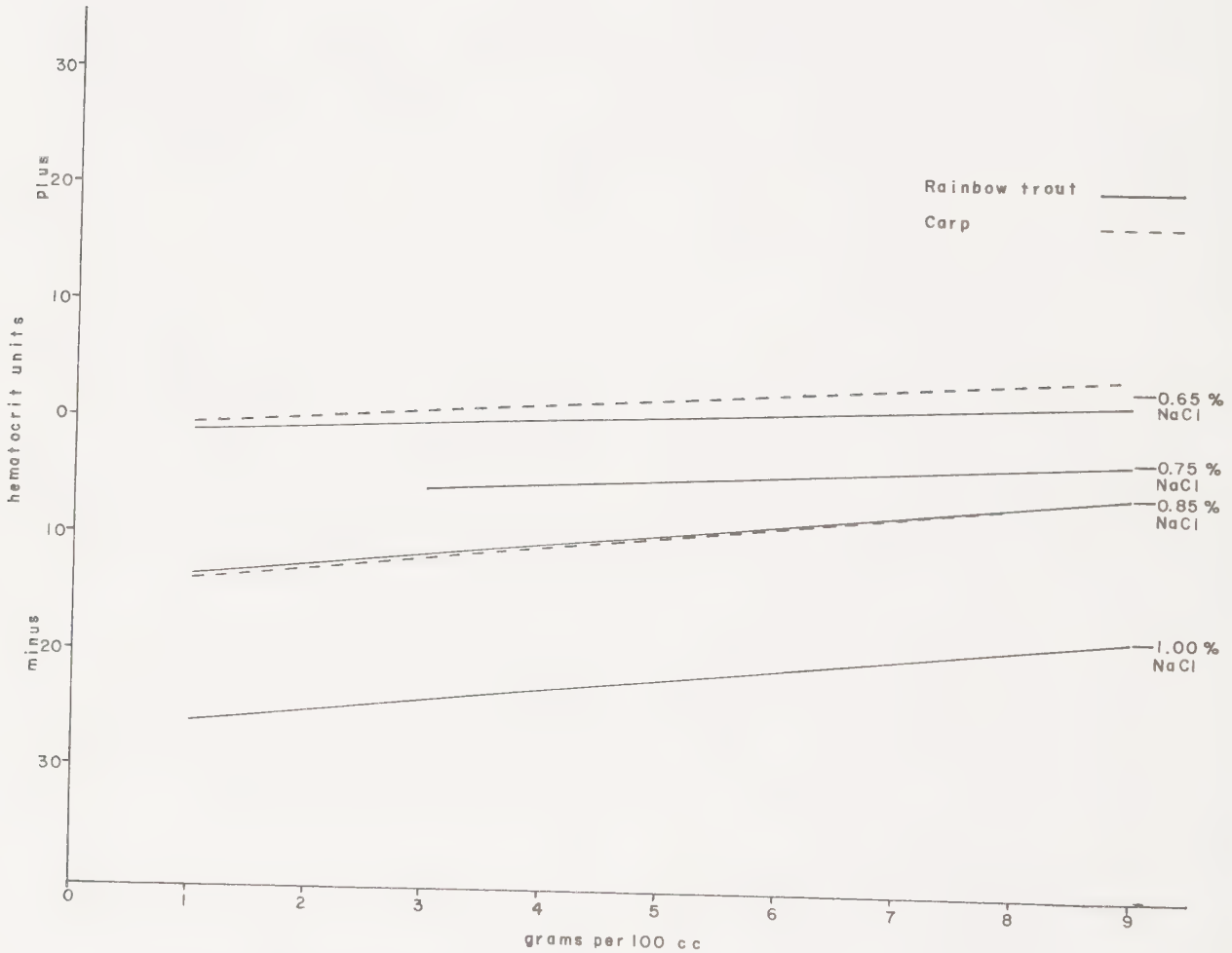

Figure 2.--Plot of hematocrit deviation against protein concentration for various levels of sodium chloride (as listed in table 3). Straight-line regression equations were computed for the data.

of sodium chloride and directly proportional to the level of protein. Suspensions of erythrocytes in salines containing 0.65- to 1.00-percent sodium chloride gave electrical readings from 4.5 units above to 27.5 units below their corresponding centrifuge values. The concentration of protein had less effect on electronic hematocrits as the level of

sodium chloride declined. The readings increased with protein concentration by 7.0 to 9.5 units at 1-percent and by 2.5 to 5.5 units at 0.65-percent sodium chloride. The difference in conductivity between erythrocytes of rainbow trout and carp were relatively small.

The deviation of electronic readings due to variations in the electrolyte content of fish blood can be estimated from Black's (1957) review of freezing points of bloods for several teleosts. The values are related to species and osmotic concentration of the external environment. The bloods of most fish in freshwater froze between -0.49 and -0.67° C. According to Hodgman, Weast, and Selby (1960), salines containing approximately 0.80- to 1.15-percent sodium chloride have similar freezing points. Phillips et al. (1956) suggested that nutritional conditions may influence the range of variability in the concentration of inorganic elements in brown trout blood. Seasonal changes in these constituents were observed in the blood of smallmouth bass by Shell (1961). Considering these data it is possible that the electronic hematocrit readings in tables 1 and 2 were affected by blood electrolytes.

The quantities of serum proteins of fish vary according to sex, reproductive condition, food, disease, oxygen depletion, and other factors (Booke, 1964). Some concentrations of protein reported per 100 cc. of blood include 1.8 to 4.5 grams for smallmouth bass (Shell, 1961), 2.01 to 2.04 grams for brown and brook trout (Phillips et al., 1957), 3.33 to 4.70 grams for rainbow trout (Meisner and Hickman, 1962), and 3.18 to 7.96 grams for brook trout (Brooke, 1964).

Assuming a physiologic level of electrolytes in the blood, electronic hematocrits could deviate 4 or 5 units depending on protein concentration. This may not be a comparatively large error, but the degree to which bovine albumin simulates blood proteins of fish with respect to conductivity is not known.

Though the regressions in figure 2 demonstrate the effect of electrolyte and protein concentration on conductivity, they do not correspond to results obtained by Kernen, Wurzel, and Okada (1961) on human blood. They found that electronic values corresponded to centrifuge hematocrits at a protein concentration of 7.4 grams per 100 cc. (bovine albumin dissolved in physiological saline). Their regression extended from -9.9 hematocrit units at 2.5 grams of protein per 100 cc. to +6.6 units at 11.0 grams per 100 cc. The inconsistency between data on humans and on fish may be related to the preparation of cellular suspensions or, perhaps, to an additional variable such as the relative size of fish erythrocytes. The red blood cells of fish are generally larger than and shaped differently from those of mammals, and it is conceivable that the comparatively low electronic hematocrits are partially correlated with differences in their insulatory characteristics (Smith, 1952; Hawk, Oser, and Summerson, 1954; and Mott, 1957). Larger cells may have less surface area per volume and have an effect on the electronic hematocrit which is analogous to that of fewer cells.

CONCLUSIONS

The YSI Electronic Hematocrit gives relatively rapid and reproducible results with fish blood. Centrifuge hematocrits greater than 25 can be estimated by correcting the electronic readings. Electronic readings were not correlated linearly with centrifuge values which were less than 25. They may be used, however, as gross but rapid indicators of anemia. Observations on the conductivities of plasma and various-strength salines suggest the instrument could be used to measure electrolyte levels in fish blood. The conductivities of blood samples changed with time and became closer to centrifuge values than the initial readings. The estimations of hematocrits from the later readings were time consuming and required heparinized blood. The electrical measurement may be influenced by variations in blood electrolytes and proteins, and by the average size of erythrocytes.

SUMMARY

Experiments with a YSI, Model 30 Electronic Hematocrit indicate that measurements of the conductivity of fish blood can be used to estimate hematocrit. The method is relatively

rapid and reproducible, but readings must be corrected to estimate centrifuge hematocrits of approximately 25 or more. The individual differences between the two methods varied widely and the differences between means of the methods deviated according to species. The electronic hematocrits do not change proportionately with centrifuge values less than 25, and are not easily corrected. The disproportionate change of electronic readings in the low hematocrit range may prove useful as a gross but rapid method for detecting anemia. Variations in serum electrolytes and proteins of fish, and in the average size of erythrocytes may interfere seriously with electrical measurements of hematocrits.

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BUREAU OF SPORT FISHERIES AND WILDLIFE
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POSTAGE AND FEES PAID
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